

## PAREVINS AND TACHYTEGRINS

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5 invention.

### Cross-Reference to Related Applications

This application is a continuation-in-part of application Serial No. 08/674,622, filed July 3, 1996, which  
10 is a continuation-in-part of provisional application Serial No. 60/000,898, filed July 6, 1995. The contents of these applications are hereby incorporated herein by references in their entirety.

### 15 Technical Field

The invention relates to the field of antibiotic peptides. In particular, the invention concerns short peptides with unique patterns of cysteine type residues and conformations that have a wide range of antimicrobial  
20 activities.

### Background Art

One of the defense mechanisms against infection by both animals and plants is the production of peptides that have  
25 antimicrobial and antiviral activity. Various classes of these peptides have been isolated from tissues of both plants and animals. PCT application WO 95/03325 published 2 February 1995 contains a review of the literature on this subject. Such peptides include tachyplesins, which are 17-18  
30 amino acid peptides containing four invariant cysteines, the defensins,  $\beta$ -defensins, and insect defensins, which are

somewhat longer peptides characterized by six invariant cysteines, and antifungal and antibacterial peptides and proteins which have been found in plants.

5 The applications in the series of which WO 95/03325 is a part provide a new class of antimicrobial and antiviral peptides, designated "protegrins", representative members of which have been isolated from porcine leukocytes. These peptides are useful as antibacterial antiviral and antifungal agents in both plants and animals.

10 The isolation of some of the protegrin peptides was reported in a paper by Kokryakov, V.N. et al. *FEBS* (1993) 337:231-236 (July issue). A later publication described the presence of a new protegrin, whose sequence and that of its precursor were deduced from its isolated cDNA clone. Zhao, C  
15 et al, *FEBS Letters* (1994) 346:285-288. An additional paper disclosing cationic peptides from porcine neutrophils was published by Mirgorodskaya, O.A. et al. *FEBS* (1993) 330:339-342. Storici, P. et al. *Biochem Biophys Res Comm* (1993) 196:1363-1367, report the recovery of a DNA sequence which  
20 encodes a pig leukocyte antimicrobial peptide with a cathelin-like prosequence. The peptide is reported to be one of the protegrins. Additional publications related to protegrins are Harwig, S.S.L., et al. *J Peptide Sci* (1995) in  
25 press; Zhao, C., et al. *FEBS Lett* (1995) 376:130-134; Zhao, C. et al. *FEBS Lett* (1995) 368:197-202. See also, U.S. Patent No. 5,464,823, U.S. Patent No. 5,696,486, WO 95/03325, WO 96/37508 and WO 98/03192.

30 The protegrins have also been found to bind to endotoxins -- i.e., the lipopolysaccharide (LPS) compositions derived from gram-negative bacteria which are believed

responsible for gram-negative sepsis. The protegrins are also effective in inhibiting the growth of organisms that are associated with sexually transmitted diseases such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

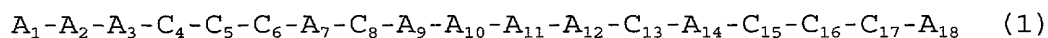
- 5       The invention described below relates to peptide type compounds that are related to the protegrins described above, but reflect displacements of the protegrin cysteines at positions 6 and 15. The availability of these compounds, the preferred forms of which are designated parevins and
- 10 tachytegrins, expands the repertoire of antimicrobial peptides and permits more exquisite matching of indications to antimicrobial formulations. Although at least one of C<sub>4</sub>, C<sub>5</sub>, C<sub>16</sub> or C<sub>17</sub> in the formula set forth below must be cysteine, the common name terminology of these components reflects
- 15 particularly preferred situations wherein both of C<sub>4</sub> and C<sub>17</sub> are cysteine type residues (the tachytegrins) or where both C<sub>5</sub> and C<sub>16</sub> are cysteine type residues (the parevins).

#### Disclosure of the Invention

- 20       The invention provides compounds which retain generally the antimicrobial activity of the protegrins discussed above, but differ in conformation due to the dislocation of the cysteine residues at positions 6 and/or 15 of these protegrins. Surprisingly, these modified compounds exhibit
- 25 activity spectra which are analogous to those of the protegrins, but offer the opportunity to fine-tune the biological activity of antibiotics and antivirals. All of these peptides can be produced synthetically and those that contain only gene-encoded amino acids can also be produced
- 30 recombinantly. These compounds are useful as preservatives

or in pharmaceutical compositions in treating or preventing infection in animals. Alternatively, the peptides can be formulated into compositions which can be applied to plants to protect them against viral or microbial infection. In  
5 still another approach, the DNA encoding the peptides can be expressed *in situ*, in animals or preferably in plants, to combat infections. The peptides are also useful as standards in antimicrobial assays and in binding endotoxins.

Accordingly, in one aspect, the invention is directed to  
10 a purified and isolated or recombinantly or synthetically produced compound which contains the amino acid sequence



15 said compound containing 11-24 amino acid residues. The sequence shown as (1) can be extended at the N and/or C terminus with non-interfering amino acids or sequence.

The compounds also include the N-terminal acylated and/or C-terminal amidated or esterified forms and may be  
20 either in the, optionally -SH stabilized, linear or in a disulfide-bridged form.

In the amino acid sequence shown, each of  $A_1-A_3$  is independently present or not present, and if present each is independently a basic, hydrophobic, polar/large, or small  
25 amino acid;

each of  $C_4, C_5, C_6, C_{15}, C_{16}$  and  $C_{17}$  is independently cysteine, homocysteine or penicillamine or a basic, hydrophobic, polar/large, or small amino acid, and  $C_4$  and/or  $C_{17}$  may be present or not present;  $C_6$  and/or  $C_{15}$  may also be  
30 acidic;

each of C<sub>8</sub> and C<sub>13</sub> is independently cysteine,  
homocysteine or penicillamine;

each of A<sub>7</sub> and A<sub>14</sub> is independently a hydrophobic or a  
small amino acid;

- 5        A<sub>9</sub>-A<sub>12</sub> must be capable of effecting a or  $\beta$ -turn when  
contained in the compound and at least one of A<sub>9</sub>-A<sub>12</sub> must be a  
basic amino acid;

A<sub>18</sub> is present or not present, and if present, is a  
basic, hydrophobic, polar/large or small amino acid.

- 10       The compounds of the invention may, in the alternative,  
contain a modified form of formula (1) wherein one or both of  
C<sub>8</sub>, and C<sub>13</sub> is independently replaced by a basic,  
hydrophobic, polar/large, acidic, or small amino acid.

- In all of the compounds of the invention at least about  
15 15% and no more than about 50% of the amino acids must be  
basic amino acids, and the compounds must have a net charge  
of +1 at physiological pH;

with the proviso that at least one of C<sub>4</sub>, C<sub>5</sub>, C<sub>16</sub> and C<sub>17</sub>  
must be cysteine, homocysteine or penicillamine; and

- 20       only one of C<sub>4</sub>, C<sub>5</sub>, and C<sub>6</sub>, and only one of C<sub>15</sub>, C<sub>16</sub> and C<sub>17</sub>  
can be cysteine, homocysteine or penicillamine.

- A particular advantage of some of the peptides of the  
invention, especially those which contain fewer amino acids,  
lies in their reduced size. As a result of this, they are  
25 less costly to produce, generally are expected to provide  
better distribution in tissue, and are less immunogenic. As  
they provide alternative structures, they are likely to have  
different pharmacokinetic and toxicological profiles.

- In still other aspects, the invention is directed to  
30 recombinant materials useful for the production of the

peptides of the invention as well as plants or animals modified to contain expression systems for the production of these peptides. The invention is also directed to pharmaceutical compositions and compositions for application  
5 to plants containing the peptides of the invention as active ingredients or compositions which contain expression systems for production of the peptides or for *in situ* expression of the nucleotide sequence encoding these peptides. The invention is also directed to methods to prepare the  
10 invention peptides synthetically, to antibodies specific for these peptides, and to the use of the peptides as preservatives.

In other aspects, the invention is directed to the use of the compounds of the invention as standards in  
15 antimicrobial assays. The compounds may also be used as antimicrobials in solutions useful in eye care, such as contact lens solutions, and in topical or other pharmaceutical compositions for treatment of sexually transmitted diseases (STDs). The invention is also directed  
20 to use of the invention compounds as preservatives for foods or other perishables. As the invention peptides can inactivate endotoxin, the invention is also directed to a method to inactivate endotoxins using the compounds of the invention and to treat gram-negative sepsis by taking  
25 advantage of this property.

#### Brief Description of the Drawings

FIGS. 1A and 1B show antibacterial activity of two of the parevins against *E. coli* ML-35p;

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FIGS. 2A and 2B show antibacterial activity of two of the parevins against *Listeria monocytogenes*;

FIGS. 3A and 3B shows antifungal activity of two of the parevins against *Candida albicans*;

5        FIGS. 4A and 4B shows antibacterial activity of a tachytegrin against *E. coli* ML-35p;

FIGS. 5A and 5B shows antibacterial activity of a tachytegrin against *B. subtilis*; and

10       FIGS. 6A and 6B shows antibacterial activity of a tachytegrin against *S. typhimurium* 14028s.

#### Modes of Carrying Out the Invention

The peptides of the invention are characterized by the amino acid sequence:

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(1)  $A_1-A_2-A_3-C_4-C_5-C_6-A_7-C_8-A_9-A_{10}-A_{11}-A_{12}-C_{13}-A_{14}-C_{15}-C_{16}-C_{17}-A_{18}$

20       and its defined modified forms. Any of these peptides which may coincidentally occur in nature must be in purified and isolated form or prepared recombinantly or synthetically.

25       The designation  $A_n$  in each case represents an amino acid at the specified position in the peptide. As defined,  $A_1-A_3$ ,  $C_4$ ,  $C_{17}$ , and/or  $A_{18}$  may or may not be present. However, the peptides of the invention contain 11-24 amino acids. Thus, the sequence shown as (1) can be extended at the N and/or C terminus with non-interfering amino acids or sequence. The positions of the cysteine, homocysteine or penicillamine residues, shown as C in formula (1), are invariant in one embodiment of the peptides of the invention; however, in the modified forms of the peptides containing the sequence of

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formula (1), also included within the scope of the invention, one or more of these cysteines may be replaced by a small, basic acidic or hydrophobic amino acid. However, at least one of C<sub>4</sub>, C<sub>5</sub>, C<sub>16</sub> and C<sub>17</sub> must be cysteine, homocysteine  
5 penicillamine.

All of the peptides of the invention, however, have a net positive charge of at least +1 at physiological pH; approximately 15%-50% of the amino acid residues included in the sequence should be basic. For embodiments having as few  
10 as 11 amino acids, there may be only one basic amino acid residue; however, at least two basic residues, even in this short-chain residue, are preferred. If the peptide contains as many as 15 amino acid residues, two basic residues are required. It is preferred that at least 20% of the amino  
15 acids in the sequence be basic, more preferably 30%, but not more than 50%.

The active peptides also preferably contain a  $\beta$ -turn bracketed by two strands that form a  $\beta$ -sheet. While not intending to be bound by any theory, applicants believe that  
20 antimicrobial activity of the compounds containing the sequence of formula (1) is associated with such a  $\beta$ -turn bracketed by two strands that form a  $\beta$ -sheet structure. The amino acids A<sub>9</sub>-A<sub>12</sub> must be capable of effecting a  $\beta$ -turn, which can be encouraged by the cystine bond between C<sub>8</sub> and C<sub>13</sub>,  
25 as well as by hydrogen bonding between A<sub>9</sub> and A<sub>12</sub>. The presence of proline at A<sub>10</sub> and/or A<sub>11</sub> does not interfere with the  $\beta$ -turn stabilized by the presence of a hydrophobic amino acid at positions A<sub>9</sub> or A<sub>12</sub>.

As used herein, " $\beta$ -turn" refers to a recognized sub-  
30 class of reverse-turns. Typically, a " $\beta$ -turn" is a four



amino acid residue peptide segment that reverses the direction of a polypeptide chain so as to allow a single polypeptide chain to adopt an anti-parallel  $\beta$ -sheet secondary structure. Generally, the two internal amino acid residues of the  $\beta$ -turn are not involved in the hydrogen-bonding of the  $\beta$ -sheet; the two amino acid residues on either side of the internal residues are included in the hydrogen-bonding of the  $\beta$ -sheet. The term " $\beta$ -turn" expressly includes all types of peptide  $\beta$ -turns commonly known in the art including, but not limited to, type-I, type-II, type-III, type-I', type-II', and type-III'  $\beta$ -turns (see, Rose et al., 1985, Adv. Protein Chem. 37:1-109; Wilmer-White et al., 1987, Trends Biochem. Sci. 12:189-192; Wilmot et al., 1988, J. Mol. Biol. 206:759-777; Tramontano et al., 1989, Proteins; Struct. Funct. Genet. 6:382-394).

The presence of the four invariant cysteines of the protegrins or of the C<sub>8</sub> and C<sub>13</sub> cysteines, homocysteine or penicillamine of the compounds of the present invention is helpful in effecting the  $\beta$ -turn conformation; however, by properly choosing the substitutions, one or both of the cysteine, homocysteine or penicillamine residues at C<sub>8</sub> or C<sub>13</sub> can be replaced without substantially disturbing the three-dimensional shape of the molecule.

The  $\beta$  sheets are believed to be effected by the sequences surrounding C<sub>8</sub> and C<sub>13</sub>, and are inclusive of these residues. Thus, in the unmodified forms of the compound, A<sub>7</sub> and A<sub>14</sub> are preferably hydrophobic amino acids. The cysteine residues may also, then, be replaced by other residues which do not affect the maintenance of the  $\beta$  sheet formation; these

substitutions would include acidic, basic, hydrophobic polar or small amino acids.

The amino terminus of the peptide may be in the free amino form or may be acylated by a group of the formula  $\text{RCO-}$ ,  
5 wherein R represents a hydrocarbyl group of 1-6C. The hydrocarbyl group is saturated or unsaturated and is typically, for example, methyl, ethyl, i-propyl, t-butyl, n-pentyl, cyclohexyl, cyclohexene-2-yl, hexene-3-yl, hexyne-4-yl, and the like.

10 The C-terminus of the peptides of the invention may be in the form of the underivatized carboxyl group, either as the free acid or an acceptable salt, such as the potassium, sodium, calcium, magnesium, or other salt of an inorganic ion or of an organic ion such as caffeine. In some embodiments,  
15 it is difficult to make salts since the remainder of the molecule bears a positive charge which may repel the relevant cation. The carboxyl terminus may also be derivatized by formation of an ester with an alcohol of the formula  $\text{ROH}$ , or may be amidated by an amine of the formula  $\text{NH}_3$ , or  $\text{RNH}_2$ , or  
20  $\text{R}_2\text{NH}$ , wherein each R is independently hydrocarbyl of 1-6C as defined above. Amidated forms of the peptides wherein the C-terminus has the formula  $\text{CONH}_2$  are preferred.

As the peptides of the invention contain substantial numbers of basic amino acids, the peptides of the invention  
25 may be supplied in the form of the acid addition salts. Typical acid addition salts include those of inorganic ions such as chloride, bromide, iodide, fluoride or the like, sulfate, nitrate, or phosphate, or may be salts of organic anions such as acetate, formate, benzoate and the like. The

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acceptability of each of such salts is dependent on the intended use, as is commonly understood.

The peptides of the invention that contain at least two cysteines, homocysteine or penicillamine may be in straight-  
5 chain or cyclic form. The straight-chain forms are convertible to the cyclic forms, and vice versa. Methods for forming disulfide bonds to create the cyclic peptides are well known in the art, as are methods to reduce disulfides to form the linear compounds. The linear compounds can be  
10 stabilized by addition of a suitable alkylating agent such as iodoacetamide.

The cyclic forms are the result of the formation of disulfide linkages among all or some of the four cysteine, homocysteine or penicillamine residues that may be present.

15 Cyclic forms of the invention include all possible permutations of disulfide bond formation; if the -SH containing amino acids are numbered in order of their occurrence starting at the N-terminus as C<sub>4</sub>, C<sub>5</sub>, C<sub>6</sub>, C<sub>8</sub>, C<sub>13</sub>, C<sub>16</sub>, C<sub>17</sub> or C<sub>18</sub>, these permutations include, when two  
20 disulfides are present:

- a) C<sub>4</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>;
- b) C<sub>4</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>;
- c) C<sub>4</sub>-C<sub>15</sub> and C<sub>8</sub>-C<sub>13</sub>;
- d) C<sub>5</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>;
- 25 e) C<sub>5</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>;
- f) C<sub>5</sub>-C<sub>15</sub> and C<sub>8</sub>-C<sub>13</sub>;
- g) C<sub>6</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>;
- h) C<sub>6</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>;
- i) C<sub>4</sub>-C<sub>8</sub> and C<sub>13</sub>-C<sub>17</sub>;
- 30 j) C<sub>4</sub>-C<sub>8</sub> and C<sub>13</sub>-C<sub>16</sub>;

k)  $C_5-C_8$  and  $C_{13}-C_{17}$ ; and

l)  $C_5-C_8$  and  $C_{13}-C_{16}$ ;

When one disulfide is present, these permutations

5 include:

$C_4-C_{17}$ ;

$C_4-C_{16}$ ;

$C_4-C_{15}$ ;

$C_5-C_{17}$ ;

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$C_5-C_{16}$ ;

$C_5-C_{15}$ ;

$C_6-C_{17}$ ;

$C_6-C_{16}$ ;

$C_8-C_{13}$ ;

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$C_4-C_8$ ;

$C_5-C_8$ ;

$C_{13}-C_{17}$ ; and

$C_{13}-C_{16}$ .

In the modified forms of the peptides, where 1 or 2  
20 cysteines, homocysteine or penicillamine are replaced,  
similar permutations are available as in when 2-3 cysteines,  
homocysteine or penicillamine are present.

The linearalized forms of the native cyclic peptides  
have valuable activities, even when chemically stabilized to  
25 preserve the sulfhydryl form of cysteine, homocysteine or  
penicillamine for example, by reaction with iodoacetamide.  
The compounds of the invention also include linearalized  
forms which are stabilized with suitable reagents. As  
defined herein, "SH-stabilized" forms of the peptides of the

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invention contain sulfhydryl groups reacted with standard reagents to prevent reformation into disulfide linkages.

An alternative approach to providing linear forms of the invention compounds comprises use of the modified form of the  
5 peptides where residues at C<sub>8</sub> and/or C<sub>13</sub> are replaced by amino acids which do not form cystine linkages, in combination with stabilization of any cysteine, homocysteine or penicillamine residues at C<sub>4</sub>, C<sub>5</sub>, or C<sub>6</sub> and/or C<sub>15</sub>, C<sub>16</sub>, C<sub>17</sub>.

Forms of the invention compounds which have only one  
10 disulfide bond are conveniently obtained by replacing the cysteine, homocysteine or penicillamine residues at C<sub>8</sub> and/or C<sub>13</sub>, preferably both, with amino acids which do not form disulfide linkages.

The amino acids denoted by A<sub>n</sub> may be those encoded by  
15 the gene or analogs thereof, and may also be the D-isomers thereof. One preferred embodiment of the peptides of the invention is that form wherein all of the residues are in the D-configuration thus conferring resistance to protease activity while retaining antimicrobial or antiviral  
20 properties. The resulting peptides are enantiomers of the native L-amino acid-containing forms.

In one class of peptides described herein, either one or both of the residues found at C<sub>5</sub> and/or C<sub>16</sub> is a basic amino acid and/or at least one of A<sub>1</sub>-A<sub>3</sub> and C<sub>4</sub> is hydrophobic and/or  
25 at least one, and preferably all four of these amino acids are deleted. By suitable manipulation of these and other features, the range of conditions under which the class of peptides of the present invention are effective can be varied. Furthermore, the spectrum of microbes against which

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they are effective can also be modified. This is further described hereinbelow.

The amino acid notations used herein are conventional and are as follows:

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Amino Acid	One-Letter Symbol	Three-Letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

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The amino acids not encoded genetically are abbreviated as indicated in the discussion below.

In the specific peptides shown in the present application, the L-form of any amino acid residue having an  
5 optical isomer is intended unless the D-form is expressly indicated by a dagger superscript (<sup>†</sup>).

The compounds of the invention are peptides which are partially defined in terms of amino acid residues of designated classes. Amino acid residues can be generally  
10 subclassified into major subclasses as follows:

Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is  
15 contained when the peptide is in aqueous medium at physiological pH.

Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface  
20 positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Hydrophobic: The residues are not charged at physiological pH and the residue is repelled by aqueous  
25 solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

Polar/large: The residues are not charged at physiological pH, but the residue is not sufficiently  
30 repelled by aqueous solutions so that it would seek inner

positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

This description also characterizes certain neutral amino acids as "small" since their side chains are not  
5 sufficiently large, even if polar groups are lacking, to confer hydrophobicity. "Small" amino acids are those with four carbons or less when at least one polar group is on the side chain and three carbons or less when not.

It is understood, of course, that in a statistical  
10 collection of individual residue molecules some molecules will be charged, and some not, and there will be an attraction for or repulsion from an aqueous medium to a greater or lesser extent. To fit the definition of "charged," a significant percentage (at least approximately  
15 25%) of the individual molecules are charged at physiological pH. The degree of attraction or repulsion required for classification as polar or nonpolar is arbitrary and, therefore, amino acids specifically contemplated by the invention have been classified as one or the other. Most  
20 amino acids not specifically named can be classified on the basis of known behavior.

Amino acid residues can be further subclassified as cyclic or noncyclic, and aromatic or nonaromatic, self-explanatory classifications with respect to the side-chain  
25 substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of four carbon atoms or less, inclusive of the carboxyl carbon, provided an additional polar substituent is present; three or less if not. Small residues are, of course, always  
30 nonaromatic.



For the naturally occurring protein amino acids, subclassification according to the foregoing scheme is as follows.

5	Acidic	Aspartic acid and Glutamic acid
	Basic	Noncyclic: Arginine, Lysine Cyclic: Histidine
	Small	Glycine, Serine, Alanine, Threonine
	Polar/large	Asparagine, Glutamine
10	Hydrophobic	Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan

The gene-encoded secondary amino acid proline is a special case due to its known effects on the secondary conformation of peptide chains, and is not, therefore, included in a group. Cysteine and other -SH containing amino acid residues are also not included in these classifications since their capacity to form disulfide bonds to provide secondary structure is critical in the compounds of the present invention.

Certain commonly encountered amino acids, which are not encoded by the genetic code, include, for example,  $\beta$ -Alanine ( $\beta$ -Ala), or other omega-amino acids, such as 3-aminopropionic, 2,3-diaminopropionic (2,3-diaP), 4-aminobutyric and so forth, 1-aminisobutyric acid (Aib), sarcosine (Sar) or N-methyl glycine (MeGly), ornithine (Orn), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), 2-naphthylalanine (2-Nal); 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic);  $\beta$ -2-thienylalanine (Thi); methionine

sulfoxide (MSO); and homoarginine (Har). These also fall conveniently into particular categories.

Based on the above definitions,

Sar or MeGly,  $\beta$ -Ala, and Aib are small;

5 t-BuA, t-BuG, N-MeIle, Nle, Mvl, Cha, Phg, Nal, Thi and Tic are hydrophobic;

Orn, 2,3-diaP and Har are basic;

Cit, Acetyl Lys, and MSO are polar/large.

The various omega-amino acids are classified according  
10 to size as small ( $\beta$ -Ala and 3-aminopropionic) or as large and hydrophobic (all others).

Other amino acid substitutions of those encoded in the gene can also be included in peptide compounds within the scope of the invention and can be classified within this  
15 general scheme according to their structure.

In all of the peptides of the invention, one or more amide linkages (-CO-NH-) may optionally be replaced with another linkage which is an isostere such as -CH<sub>2</sub>NH-, -CH<sub>2</sub>S-, -CH<sub>2</sub>CH<sub>2</sub>-, -CH=CH- (cis and trans), -COCH<sub>2</sub>-, -CH(OH)CH<sub>2</sub>- and  
20 -CH<sub>2</sub>SO-. This replacement can be made by methods known in the art. The following references describe preparation of peptide analogs which include these alternative-linking moieties: Spatola, A.F., *Vega Data* (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review);  
25 Spatola, A.F., in Chemistry and Biochemistry of Amino Acids Peptides and Proteins, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983) (general review); Morley, J.S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D., et al., *Int J Pept Prot Res* (1979) **14**:177-185 (-CH<sub>2</sub>NH-,  
30 -CH<sub>2</sub>CH<sub>2</sub>-); Spatola, A.F., et al., *Life Sci* (1986) **38**:1243-1249

5 (-CH<sub>2</sub>-S); Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G., et al., *J Med Chem* (1980) **23**:1392-1398 (-COCH<sub>2</sub>-); Jennings-White, C., et al., *Tetrahedron Lett* (1982) **23**:2533 (-COCH<sub>2</sub>-); Szelke, M., et al., European Application EP 45665 (1982) CA:**97**:39405 (1982) (-CH(OH)CH<sub>2</sub>-); Holladay, M.W., et al., *Tetrahedron Lett* (1983) **24**:4401-4404 (-C(OH)CH<sub>2</sub>-); and Hruby, V.J., *Life Sci* (1982) **UB**:189-199 (-CH<sub>2</sub>-S-).

10 In addition to analogs which contain isosteres in place of peptide linkages, the peptides or proteins of the invention include peptide mimetics in general, such as those described by Olson, G.L. et al. *J Med Chem* (1993) **36**:3039-3049 and retro-inverso type peptides as described by Chorev, M. et al. *Science* (1979) **204**:1210-1212; and Pallai, P.V. et al., *Int J Pept Protein Res* (1983) **21**:84-92.

15 One class of preferred embodiments of the compounds invention includes the "unmodified" forms where positions 8 and 13 are independently cysteine, homocysteine or penicillamine residues, especially in the disulfide bonded form.

In addition, or alternatively, each of A<sub>7</sub> and A<sub>14</sub> is a hydrophobic acid, preferably Ile, Val, Leu, NLe, Trp, Tyr or Phe or is a small amino acid, Ala, Gly, Ser or Thr.

25 In another set of preferred embodiments, all of A<sub>1</sub>-A<sub>3</sub> are not present or at least one, and preferably two of A<sub>1</sub>-A<sub>3</sub> is a hydrophobic amino acid, preferably Ile, Val, Leu, NLe, Trp, Tyr or Phe.

30 In another set of preferred embodiments, C<sub>4</sub> and/or C<sub>17</sub> is not present or, if present, is a cysteine, homocysteine or penicillamine or a hydrophobic amino acid, preferably Ile,

Val, Leu, NLe, Trp, Tyr or Phe, or a small amino acid, preferably S, A, G or T.

In another set of preferred embodiments, C<sub>5</sub> and/or C<sub>16</sub> is a cysteine, homocysteine or penicillamine or a hydrophobic amino acid, preferably Ile, Val, Leu, NLe, Trp, Tyr or Phe, or a small amino acid, preferably S, A, G or T.

In another set of preferred embodiments, A<sub>9</sub>-A<sub>12</sub> contain at least one hydrophobic amino acid residue, preferably Phe, Tyr or Trp.

Other preferred embodiments include those wherein each of A<sub>1</sub> and A<sub>9</sub> is independently selected from the group consisting of R, K and Har; more preferably, both A<sub>1</sub> and A<sub>9</sub> are R; however, each of A<sub>1</sub> may be absent.

In another class of preferred embodiments, each of A<sub>2</sub> and A<sub>3</sub> is independently selected from the group consisting of G, A, S and T; more preferably, A<sub>2</sub> and A<sub>3</sub> are G; however, A<sub>2</sub> and/or A<sub>3</sub> may be absent.

In another set of preferred embodiments, one of A<sub>9</sub> and A<sub>12</sub> is R, K, Har, Orn or H preferably R and the other is I, V, L, NLe, W, Y or F, preferably R, F or W, or is S, G, A or T.

In another set of preferred embodiments, each of A<sub>10</sub> and A<sub>11</sub> is independently proline or a small, basic or hydrophobic amino acid, preferably R, G, W or P.

A<sub>18</sub> is preferably absent, but when present, is preferably R, K or Har, most preferably R.

Also preferably when all four amino acids A<sub>1</sub>-A<sub>3</sub> and C<sub>4</sub> are present, A<sub>1</sub> is basic, C<sub>4</sub> is C or basic, and A<sub>2</sub> and A<sub>3</sub> are small amino acids, or at least one of A<sub>1</sub>-A<sub>3</sub> and C<sub>4</sub> is hydrophobic. Preferred embodiments of A<sub>1</sub>-A<sub>3</sub> include R-G-G, K-G-S, K-S-G, and the like.

As described above, the compounds of Formula (1) are either in cyclic or noncyclic (linearalized) form or may be modified wherein 1 or 2 of the cysteines, homocysteine or penicillamine at C<sub>8</sub> and C<sub>13</sub> are replaced by a small,

- 5 hydrophobic, or a basic amino acid residue. Such modification is preferred when compounds containing only one disulfide bond are prepared. If the linearalized forms of the compound of Formula (1) are prepared, or if linearalized forms of those modified peptides which contain at least two
- 10 cysteines are prepared, it is preferred that the sulphydryl groups be stabilized by addition of a suitable reagent. Preferred embodiments for the hydrophobic amino acid to replace cysteine, homocysteine or penicillamine residues at C<sub>8</sub> and/or C<sub>13</sub> are I, V, L and Nle, preferably I, V or L.
- 15 Preferred small amino acids to replace the cysteine, homocysteine or penicillamine residues include G, A, S and T, more preferably G. Preferred basic amino acids are R and K.

Where the compounds of the invention have two disulfide bridges, particularly preferred are the pairs of bridges:

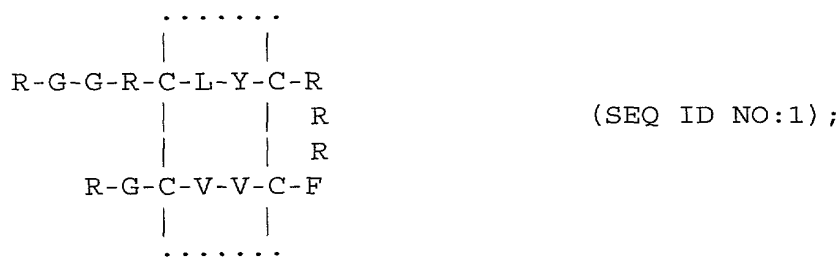
- 20 a) C<sub>5</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>;  
b) C<sub>5</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>;  
c) C<sub>5</sub>-C<sub>8</sub> and C<sub>13</sub>-C<sub>16</sub>;  
d) C<sub>4</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>; and  
e) C<sub>4</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>.
- 25 Especially preferred are the bridges:  
C<sub>5</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>; and  
C<sub>4</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>.

Where the compound has only one disulfide bridge, particularly preferred are:

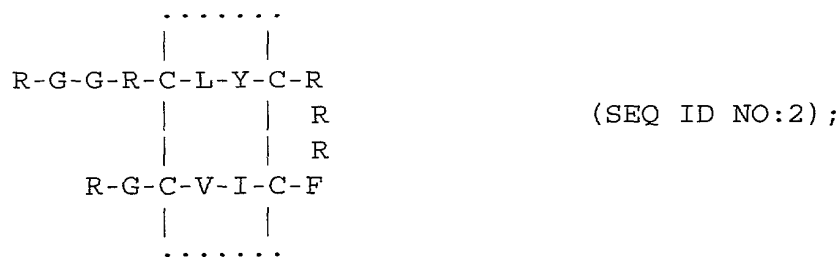
- 30 C<sub>4</sub>-C<sub>17</sub> or C<sub>5</sub>-C<sub>16</sub>.

Particularly preferred compounds of the invention, including the N-terminal acylated and C-terminal amidated forms thereof are the parevins, wherein C<sub>5</sub> and C<sub>16</sub> are both cysteine, homocysteine or penicillamine and the tachygrins wherein both C<sub>4</sub> and C<sub>17</sub> are cysteine, homocysteine or penicillamine. Also preferred are the disulfide forms of these compounds, cis-parevins wherein the two disulfide bridges are C<sub>5</sub>-C<sub>8</sub> and C<sub>13</sub>-C<sub>16</sub>; the trans-parevins wherein the disulfide bridges are C<sub>5</sub>-C<sub>16</sub> and C<sub>8</sub>-C<sub>13</sub>; and the trans-tachygrins wherein the disulfide bridges are C<sub>4</sub>-C<sub>17</sub> and C<sub>8</sub>-C<sub>13</sub>. Particularly preferred are the following parevins and tachygrins:

Parevin 1            trans \_\_\_\_\_ and cis.....

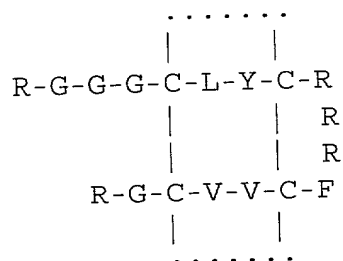


Parevin 2            trans \_\_\_\_\_ and cis.....



Parevin 3 trans\_\_\_\_\_ and cis.....

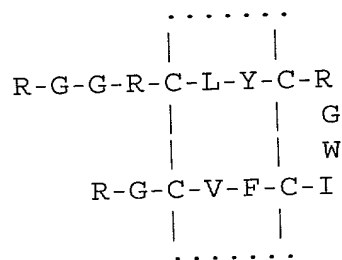
5



(SEQ ID NO:3);

Parevin 4 trans\_\_\_\_\_ and cis.....

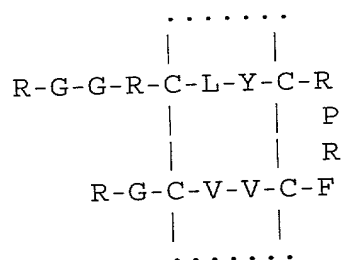
10



(SEQ ID NO:4);

15

Parevin 5 trans\_\_\_\_\_ and cis.....

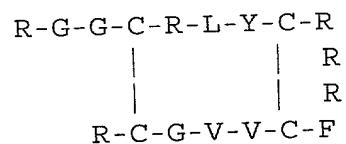


(SEQ ID NO:5);

20

Tachytegrin-1 trans\_\_\_\_\_

25



(SEQ ID NO:6);

30

[illegible]
$$\begin{array}{ccccccc} \text{R-G-G-C-R-L-Y-C-R} & & & & & & \\ | & & & & | & & \text{R} \\ | & & & & | & & \text{R} \\ \text{R-C-G-V-I-C-F} & & & & & & \end{array}$$

10

$$\begin{array}{ccccccc} \text{R-G-G-C-G-L-Y-C-R} & & & & & & \\ & | & & & | & & \text{R} \\ & | & & & | & & \text{R} \\ & & \text{R-C-G-V-V-C-F} & & & & \end{array}$$

15

Tachytegrin-4 trans\_\_\_\_\_

R-G-G-C-R-L-Y-C-R  
 |                    | G  
 |                    | W  
 R-C-G-V-F-C-I

20

Tachytegrin-5 trans\_\_\_\_\_

$$\begin{array}{ccccccc} \text{R-G-G-C-R-L-Y-C-R} & & & & & & \\ | & & & & & & \text{P} \\ | & & & & & & \text{R} \\ | & & & & & & \\ \text{R-C-G-V-V-C-F} & & & & & & \end{array}$$

25

Particularly preferred are cis- and trans-parevin-1 and trans-tachytegrin-1.

30 Typical compounds of the invention include the linear and cyclic (where possible) forms of the following compounds:



	RGGRCLYCRRRFCVVCGR	(SEQ ID NO:11);
	RGGCRLYCRRRFCVVGCR	(SEQ ID NO:12);
	RGGRCLYCRRRFCIVCG	(SEQ ID NO:13);
	RGGCRLYCRRRFCIVGC	(SEQ ID NO:14);
5	RGGGCLYCRRRFCVVCGR	(SEQ ID NO:15);
	RGGCGLYCRRRFCVVGCR	(SEQ ID NO:16);
	RGGRCLYCRGWICFVCGR	(SEQ ID NO:17);
	RGGCRLYCRGWICFVGCR	(SEQ ID NO:18);
	RGGRCLYCRPRFCVVCGR	(SEQ ID NO:19);
10	RGGCRLYCRPRFCVVGCR	(SEQ ID NO:20);
	RGGRCVYCRRRFCVVCGR	(SEQ ID NO:21);
	RGGCRVYCRRRFCVIGC	(SEQ ID NO:22);
	KGGRCLYCRRRFCVVCGR	(SEQ ID NO:23);
	KGGCRIYCRRRFCVIGC	(SEQ ID NO:24);
15	RGGXCLYCRRRFCVVC	(SEQ ID NO:25);
	RGGCXLYCRRRFCVIC	(SEQ ID NO:26);
	RGGXCLYCXR RFCVVCGR	(SEQ ID NO:27);
	RGGCXLYCXRRFCVIGCR	(SEQ ID NO:28);
	RGGRCVYCRXRFCVVCGR	(SEQ ID NO:29);
20	RGGCRVYCRXRFCVVGCR	(SEQ ID NO:30);
	RGGRCLYCRKKWCVVCGR	(SEQ ID NO:31);
	RGGCRLYCRKKWCVVGCR	(SEQ ID NO:32);
	RGGRCLYCRXRYCVVCGR	(SEQ ID NO:33);
	RGGCRLYCRXRYCVVACR	(SEQ ID NO:34);
25	RSGCLYCRRKWCVVCGR	(SEQ ID NO:35);
	RSGCLYCRRKWCVVGCR	(SEQ ID NO:36);
	RATRCIFCRRRFCVVCGR	(SEQ ID NO:37);
	RATCRIFCRRRFCVIGCR	(SEQ ID NO:38);
	RGGKCVYCRXRFCVVCGR	(SEQ ID NO:39);
30	RGGCKVYCRXRFCVIGCR	(SEQ ID NO:40);

	RATRCIFCrRRFCVVCGr	(SEQ ID NO:41);
	RATCRIFCrRRFCVVGCr	(SEQ ID NO:42);
	RGGKCVYCRxRFCVVCGR	(SEQ ID NO:43);
	RGGCKVYCRxRFCVVGCR	(SEQ ID NO:44);
5	rggrclycrrrrfcvvvgr	(SEQ ID NO:45);
	rggcrlycrrrrfcvvgr	(SEQ ID NO:46);
	rggrclycrrrrfcivcg	(SEQ ID NO:47);
	rggcrlycrrrrfcivgc	(SEQ ID NO:48);
	rgggclycrrrrfcvvvgr	(SEQ ID NO:49);
10	rggcglycrrrrfcvvgr	(SEQ ID NO:50);
	rggrclycrgwicfvvgr	(SEQ ID NO:51);
	rggcrlycrgwicfvgr	(SEQ ID NO:52);
	RGGCLRYCRPRFCVRVCR	(SEQ ID NO:53);
	RGGCRLYCRRRFCVVGCR	(SEQ ID NO:54);
15	RGVCLRYCRGRFCVRLCR	(SEQ ID NO:55);
	RGRVCLRYCRGRFCVRLCFR	(SEQ ID NO:56);
	RWRVCLRYCRGRFCVRLCLR	(SEQ ID NO:57);
	RGWRVCLKYCRGRFCVKLCLR	(SEQ ID NO:58);
	RGGRVCLRYCRGKFCVRLCLR	(SEQ ID NO:59);
20	RGGRCLYARRRFAVVCGR	(SEQ ID NO:60);
	RGGRCLYARRRFSIVC	(SEQ ID NO:61);
	RGGGCLYSRRRFAVVCGR	(SEQ ID NO:62);
	RGGRCLYARRRFGVVC	(SEQ ID NO:63);
	KGGRCLYVRRRFIVVC	(SEQ ID NO:64);
25	RGGXCLYARRRFVGCV	(SEQ ID NO:65);
	RGGXCLYAXRRFSVVCr	(SEQ ID NO:66);
	RGGCXLYAXRRFSVVGCR	(SEQ ID NO:67);
	RGGRCVYVRXRFLVCVGR	(SEQ ID NO:68);
	RGGRCLYSRKKWAVSCGR	(SEQ ID NO:69);
30	RGGRCLYSRXRYSVICGR	(SEQ ID NO:70);



RGGCGLYCRRRFCVCVGR (SEQ ID NO:101);

RGGGLCYCRRRFCVVCGR (SEQ ID NO:102);

RGGGLCYCRRRFCVVGCR (SEQ ID NO:103);

and N-terminal acylated and C-terminal amidated or  
5 esterified forms thereof, where uppercase letters represent  
L-amino acids, lower case letters represent D-amino acids, X  
is Har, x is D-Har and Z is MeGly.

Particularly preferred are the cyclic forms (where  
possible) and C-terminal amidated forms of the above  
10 compounds, especially the cyclic forms (where possible) and  
C-terminal amidated forms of:

RGGCLRYCPRRFCVRVCR (SEQ ID NO:53);

RGGCLRYCRRRFCVVGCR (SEQ ID NO:54);

15 RGVCLRYCGRFCVRLCR (SEQ ID NO:55);

RGRVCLRYCGRFCVRLCFR (SEQ ID NO:56);

RWRVCLRYCGRFCVRLCLR (SEQ ID NO:57);

RGWRVCLKYCRGRFCVKLCLR (SEQ ID NO:58);

RGGRVCLRYCRGKFCVRLCLR (SEQ ID NO:59);

20 RGGCLRYAVPRFAVRVCR (SEQ ID NO:77);

RGGCLRYTKPKFTVRVCR (SEQ ID NO:78);

RGGCLRYAVGRFAVRVCR (SEQ ID NO:79);

RGGCLRYARZRFAVRVCR (SEQ ID NO:80);

RGFCLRYTVPRFTVRFCVR (SEQ ID NO:81);

25 RGFCLRYKVGRFKVRFCVR (SEQ ID NO:82);

RGFCLRYZVGRFZVRFCVR (SEQ ID NO:83);

RGGCLRYARZRFAVRVCR (SEQ ID NO:84); and

RGGCLRYAVGRFAVRVCR (SEQ ID NO:85),

where upper and lower case letters and X and Z are as  
30 previously defined.

### Preparation of the Invention Compounds

The invention compounds are essentially peptide backbones which may be modified at the N- or C-terminus and also may contain one or two cystine disulfide linkages. The peptides may first be synthesized in noncyclized form. These peptides may then be converted to the cyclic peptides if desired by standard methods of cystine bond formation. As applied to the compounds herein, "cyclic forms" refers to those forms which contain cyclic portions by virtue of the formation of disulfide linkages between cysteine residues in the peptide. If the straight-chain forms are preferred, it is preferable to stabilize the sulfhydryl groups for any peptides of the invention which contain two or more cysteine residues.

Standard methods for synthesis of peptides can be used. Most commonly used currently are solid phase synthesis techniques; indeed, automated equipment for systematically constructing peptide chains can be purchased. Solution phase synthesis can also be used but is considerably less convenient. When synthesized using these standard techniques, amino acids not encoded by the gene and D-enantiomers can be employed in the synthesis. Thus, one very practical way to obtain the compounds of the invention is to employ these standard chemical synthesis techniques.

In addition to providing the peptide backbone, the N- and/or C-terminus can be derivatized, again using conventional chemical techniques. The compounds of the invention may optionally contain an acyl group, preferably an acetyl group at the amino terminus. Methods for acetylating or, more generally, acylating, the free amino group at the

N-terminus are generally known in the art; in addition, the N-terminal amino acid may be supplied in the synthesis in acylated form.

- At the carboxy terminus, the carboxyl group may, of course, be present in the form of a salt; in the case of pharmaceutical compositions this will be a pharmaceutically acceptable salt. Suitable salts include those formed with inorganic ions such as  $\text{NH}_4^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ , and the like as well as salts formed with organic cations such as those of caffeine and other highly substituted amines. However, when the compound of formula 1 contains a multiplicity of basic residues, salt formation may be difficult or impossible. The carboxy terminus may also be esterified using alcohols of the formula ROH wherein R is hydrocarbyl (1-6C) as defined above. Similarly, the carboxy terminus may be amidated so as to have the formula  $-\text{CONH}_2$ ,  $-\text{CONHR}$ , or  $-\text{CONR}_2$ , wherein each R is independently hydrocarbyl (1-6C) as herein defined. Techniques for esterification and amidation as well as neutralizing in the presence of base to form salts are all standard organic chemical techniques.

If the peptides of the invention are prepared under physiological conditions, the side-chain amino groups of the basic amino acids will be in the form of the relevant acid addition salts.

- Formation of disulfide linkages, if desired, is conducted in the presence of mild oxidizing agents. Chemical oxidizing agents may be used, or the compounds may simply be exposed to the oxygen of the air to effect these linkages. Various methods are known in the art. Processes useful for disulfide bond formation have been described by Tam, J.P. et

al., Synthesis (1979) 955-957; Stewart, J.M. et al., "Solid Phase Peptide Synthesis" 2d Ed. Pierce Chemical Company Rockford, IL (1984); Ahmed A.K. et al., J Biol Chem (1975) 250:8477-8482 and Pennington M.W. et al., Peptides 1990, E.

5 Giralt et al., ESCOM Leiden, The Netherlands (1991) 164-166. An additional alternative is described by Kamber, B. et al., Helv Chim Acta (1980) 63:899-915. A method conducted on solid supports is described by Albericio Int J Pept Protein Res (1985) 26:92-97.

10 A particularly preferred method is solution oxidation using molecular oxygen. This method has been used by the inventors herein to refold the compounds of the invention.

If the peptide backbone is comprised entirely of gene-encoded amino acids, or if some portion of it is so composed, 15 the peptide or the relevant portion may also be synthesized using recombinant DNA techniques. The DNA encoding the peptides of the invention may itself be synthesized using commercially available equipment; codon choice can be integrated into the synthesis depending on the nature of the 20 host.

Synthesized and recombinantly produced forms of the compounds may require subsequent derivatization to modify the N- and/or C-terminus and, depending on the isolation 25 procedure, to effect the formation of cystine bonds as described hereinabove. Depending on the host organism used for recombinant production, some or all of these conversions may already have been effected.

For recombinant production, the DNA encoding the peptides of the invention is included in an expression system 30 which places these coding sequences under control of a

suitable promoter and other control sequences compatible with an intended host cell. Types of host cells available span almost the entire range of the plant and animal kingdoms.

Thus, the compounds of the invention could be produced in  
5 bacteria or yeast (to the extent that they can be produced in a nontoxic or refractile form or utilize resistant strains) as well as in animal cells, insect cells and plant cells. Indeed, modified plant cells can be used to regenerate plants containing the relevant expression systems so that the  
10 resulting transgenic plant is capable of self protection vis-à-vis these infective agents.

The compounds of the invention can be produced in a form that will result in their secretion from the host cell by fusing to the DNA encoding the peptide, a DNA encoding a  
15 suitable signal peptide, or may be produced intracellularly. They may also be produced as fusion proteins with additional amino acid sequence which may or may not need to be subsequently removed prior to the use of these compounds as antimicrobials or antivirals.

20 Thus, the compounds of the invention can be produced in a variety of modalities including chemical synthesis, recombinant production, isolation from natural sources, or some combination of these techniques.

Any members of the invention class which coincidentally  
25 occur naturally must be supplied in purified and isolated form. By "purified and isolated" is meant free from the environment in which the peptide normally occurs (in the case of such naturally occurring peptides) and in a form where it can be used practically. Thus, "purified and isolated" form  
30 means that the peptide is substantially pure, i.e., more than



90% pure, preferably more than 95% pure and more preferably more than 99% pure or is in a completely different context such as that of a pharmaceutical preparation.

## 5        Antibodies

Antibodies to the peptides of the invention may also be produced using standard immunological techniques for production of polyclonal antisera and, if desired, immortalizing the antibody-producing cells of the immunized

10 host for sources of monoclonal antibody production.

Techniques for producing antibodies to any substance of interest are well known. It may be necessary to enhance the immunogenicity of the substance, particularly as here, where the material is only a short peptide, by coupling the hapten

15 to a carrier. Suitable carriers for this purpose include substances which do not themselves produce an immune response in the mammal to be administered the hapten-carrier conjugate. Common carriers used include keyhole limpet hemocyanin (KLH), diphtheria toxoid, serum albumin, and the  
20 viral coat protein of rotavirus, VP6. Coupling of the hapten to the carrier is effected by standard techniques such as contacting the carrier with the peptide in the presence of a dehydrating agent such as dicyclohexylcarbodiimide or through the use of linkers such as those available through Pierce  
25 Chemical Company, Chicago, IL.

The peptides of the invention in immunogenic form are then injected into a suitable mammalian host and antibody titers in the serum are monitored. It should be noted, however, that some forms of the peptides require modification  
30 before they are able to raise antibodies, due to their

resistance to antigen processing. For example, peptides containing two cystine bridges may be nonimmunogenic when administered without coupling to a larger carrier and may be poor immunogens even in the presence of potent adjuvants and  
5 when coupled in certain formats such as using glutaraldehyde or to KLH. Any lack of immunogenicity may therefore result from resistance to processing to a linear form that can fit in the antigen-presenting pocket of the presenting cell. Immunogenicity of these forms of the peptides can be enhanced  
10 by cleaving the disulfide bonds.

Polyclonal antisera may be harvested when titers are sufficiently high. Alternatively, antibody-producing cells of the host such as spleen cells or peripheral blood lymphocytes may be harvested and immortalized. The  
15 immortalized cells are then cloned as individual colonies and screened for the production of the desired monoclonal antibodies.

Recombinant techniques are also available for the production of antibodies, and thus, the antibodies of the  
20 invention include those that can be made by genetic engineering techniques. For example, single-chain forms, such as  $F_v$  forms, chimeric antibodies, and antibodies modified to mimic those of a particular species, such as humans, can be produced using standard methods. Thus, the  
25 antibodies of the invention can be prepared by isolating or modifying the genes encoding the desired antibodies and producing these through expression in recombinant host cells, such as CHO cells.

The antibodies of the invention are, of course, useful  
30 in immunoassays for determining the amount or presence of the

peptides. Such assays are essential in quality controlled production of compositions containing the peptides of the invention. In addition, the antibodies can be used to assess the efficacy of recombinant production of the peptides, as  
5 well as screening expression libraries for the presence of peptide encoding genes.

Compositions Containing the Invention Peptides and  
Methods of Use

10 The peptides of the invention are effective in inactivating a wide range of microbial and viral targets, including gram-positive and gram-negative bacteria, yeast, protozoa and certain strains of virus. Accordingly, they can be used in disinfectant compositions and as preservatives for  
15 materials such as foodstuffs, cosmetics, medicaments, or other materials containing nutrients for organisms. For use in such contexts, the peptides are supplied either as a single peptide, in admixture with several other peptides of the invention, or in admixture with additional antimicrobial  
20 agents or both. In general, as these are preservatives in this context, they are usually present in relatively low amounts, of less than 5%, by weight of the total composition, more preferably less than 1%, still more preferably less than 0.1%.

25 The peptides of the invention are also useful as standards in antimicrobial assays and in assays for determination of capability of test compounds to bind to endotoxins such as lipopolysaccharides.

For use as antimicrobials or antivirals for treatment of  
30 animal subjects, the peptides of the invention can be

formulated as pharmaceutical or veterinary compositions. Depending on the subject to be treated, the mode of administration, and the type of treatment desired -- e.g., prevention, prophylaxis, therapy; the invention peptides are  
5 formulated in ways consonant with these parameters. A summary of such techniques is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA.

The peptides of the invention can also be used as active  
10 ingredients in pharmaceutical compositions useful in treatment of sexually transmitted diseases, including those caused by *Chlamydia trachomatis*, *Treponema pallidum*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, Herpes simplex type 2 and HIV. Topical formulations are preferred and  
15 include creams, salves, oils, powders, gels and the like. Suitable topical excipients are well known in the art and can be adapted for particular uses by those of ordinary skill.

In general, for use in treatment or prophylaxis of STDs, the peptides of the invention may be used alone or in  
20 combination with other antibiotics such as erythromycin, tetracycline, macrolides, for example azithromycin and the cephalosporins. Depending on the mode of administration, the peptides will be formulated into suitable compositions to permit facile delivery to the affected areas. The  
25 tachygrins may be used in forms containing one or two disulfide bridges or may be in linear form. In addition, use of the enantiomeric forms containing all D-amino acids may confer advantages such as resistance to those proteases, such as trypsin and chymotrypsin, to which the peptides containing  
30 L-amino acids are less resistant.

The peptides of the invention can be administered singly or as mixtures of several peptides or in combination with other pharmaceutically active components. The formulations may be prepared in a manner suitable for systemic  
5 administration or topical or local administration. Systemic formulations include those designed for injection (e.g., intramuscular, intravenous or subcutaneous injection) or may be prepared for transdermal, transmucosal, or oral administration. The formulation will generally include a  
10 diluent as well as, in some cases, adjuvants, buffers, preservatives and the like. The tachytegrins can be administered also in liposomal compositions or as microemulsions.

If administration is to be oral, the peptides of the  
15 invention must be protected from degradation in the stomach using a suitable enteric coating. This may be avoided to some extent by utilizing amino acids in the D-configuration, thus providing resistance to protease. However, the peptide is still susceptible to hydrolysis due to the acidic  
20 conditions of the stomach; thus, some degree of enteric coating may still be required.

The peptides of the invention also retain their activity against microbes in the context of borate solutions that are commonly used in eye care products. Also, it is important  
25 that the peptides retain their activity under physiological conditions including relatively high saline and in the presence of serum. In addition, the peptides are dramatically less cytotoxic with respect to the cells of higher organisms as compared with their toxicity to microbes.

30

These properties, make them particularly suitable for *in vivo* and therapeutic use.

By appropriately choosing the member or members of the peptide class of the invention, it is possible to adapt the  
5 antimicrobial activity to maximize its effectiveness with respect to a particular target microbe. As used herein, "microbe" will be used to include not only yeast, bacteria, and other unicellular organisms, but also viruses. The particular peptide used can also be chosen to be advantageous  
10 in a particular context, such as low salt or physiological salt, the presence or human serum, or conditions that mimic the conditions found in blood and tissue fluids.

The peptides of the invention may also be applied to plants or to their environment to prevent virus- and microbe-  
15 induced diseases in these plants. Suitable compositions for this use will typically contain a diluent as well as a spreading agent or other ancillary agreements beneficial to the plant or to the environment.

Thus, the peptides of the invention may be used in any  
20 context wherein an antimicrobial and/or antiviral action is required. This use may be an entirely *in vitro* use, or the peptides may be administered to organisms.

In addition, the antimicrobial or antiviral activity may be generated *in situ* by administering an expression system  
25 suitable for the production of the peptides of the invention. Such expression systems can be supplied to plant and animal subjects using known techniques. For example, in animals, pox-based expression vectors can be used to generate the peptides *in situ*. Similarly, plant cells can be transformed  
30 with expression vectors and then regenerated into whole

plants which are capable of their own production of the peptides.

The peptides of the invention are also capable of inactivating endotoxins derived from gram-negative bacteria -- i.e., lipopolysaccharides (LPS) and may be used under any circumstances where inactivation of LPS is desired. One such situation is in the treatment or amelioration of gram-negative sepsis.

#### 10      Conditions Relevant to Antimicrobial/Antiviral Activity

It has been stated above that as used herein "antimicrobial" activity refers to inhibition with respect both to traditional microorganisms and to viruses, although occasionally, "antimicrobial" and "antiviral" are both specifically indicated.

Media for testing antimicrobial activity are designed to mimic certain specific conditions. The standard buffer medium, medium A, uses an underlay agar with the following composition: 0.3 mg/ml of trypticase soy broth powder, 1% w/v agarose and 10 mM sodium phosphate buffer (final pH 7.4). This will be designated either "medium A" or "standard in vitro conditions" herein.

All of the remaining media contain these same components. However, in addition:

25      A second medium contains 100 mM NaCl in order to mimic the salt levels in blood and tissue fluids. This will be designated "medium B" or "salt medium" herein.

A third medium is supplemented with 2.5% normal human serum; however, it is of low ionic strength and thus does not

30

mimic body fluids. This medium will be designated "medium C" or "serum-containing medium" herein.

A fourth medium contains 80% RPMI-1640, a standard tissue culture medium which contains the principal ions and amino acids found in blood and tissue fluids. In addition, it contains 2.5% normal human serum. This will be designated "medium D" or "physiological medium" herein.

Particularly preferred is the amidated form of this peptide.

10

#### Summary

The peptides of the invention therefore represent a peculiarly useful class of compounds because of the following properties:

15        1) They have an antimicrobial effect with respect to a broad spectrum of target microbial systems, including viruses, including retroviruses, bacteria, fungi, yeast and protozoa.

20        2) Their antimicrobial activity is effective under physiological conditions - i.e., physiological saline and in the presence of serum.

3) They are much less toxic to the cells of higher organisms than to microbes.

25        4) They can be prepared in nonimmunogenic form thus extending the number of species to which they can be administered.

5) They can be prepared in forms which are resistant to certain proteases suggesting they are antimicrobial even in lysosomes.

30



6) They can be prepared in forms that resist degradation when autoclaved, thus simplifying their preparation as components of pharmaceuticals.

7) They can be modified in amino acid sequence so as  
5 to optimize the specificity with respect to target.

8) They can be modified structurally so as to accommodate the conditions under which antimicrobial activity is to be exhibited.

10 The following examples are intended to illustrate but not to limit the invention.

#### Example 1

##### Synthesis of the Invention Compounds

15 The peptides of the invention are synthesized using conventional Fmoc chemistry on solid-phase supports. The crude synthetic peptides are refolded, purified and characterized as follows.

The crude synthetic peptide is reduced by adding an  
20 amount of dithiothreitol (DTT) equal in weight to that of the synthetic peptide, which has been dissolved at 10 mg/ml in a solution containing 6M guanidine HCl, 0.5M Tris buffer and 2 mmol EDTA, pH 8.05 and incubated for 2 hours at 52°C under nitrogen. The mixture is passed through a 0.45 um, filter,  
25 acidified with 1/20 v/v glacial acetic acid and subjected to conventional RP HPLC purification with a C18 column.

The HPLC-purified, reduced peptides are partially concentrated by vacuum centrifugation in a Speed Vac and allowed to fold for 24 hours at room temperature and air.  
30 The folding is accomplished in 0.1M Tris, pH 7.7 at 0.1 mg

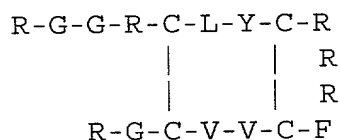
peptide/ml to minimize formation of interchain cystine disulfides. The folded compounds are concentrated and acidified with 5% acetic acid. The purity of the final products is verified by AU-PAGE, analytical HPLC and FAB-mass

5 spec.

Using this procedure, the compounds trans-parevin-1 (or the "hairpin" isoform), cis-parevin-1 (or "cloverleaf" isoform) and trans-tachytegrin-1 were prepared. These compounds are of the formulas

10

Trans-parevin-1:



(SEQ ID NO:104);

15

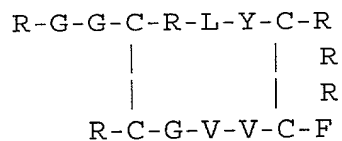
Cis-parevin-1:



(SEQ ID NO:126);

20

Trans-tachytegrin-1:



(SEQ ID NO:6);

25

30

## Example 2

### Antimicrobial Activity

The radial diffusion assay in agarose gels is conducted using radiodiffusion and gel overlay techniques as described by Lehrer, R.I. et al. J Immunol Meth (1991) 137:167-173. Briefly, the underlay agars used for all organisms had a final pH of 7.4 and contained 10 mM sodium phosphate buffer, 1% w/v agarose and 0.30 ug/ml trypticase soy broth powder (BBL Cockeysville, MD). In some cases, the underlay was supplemented with 100 mM NaCl. The units of activity in the radial diffusion assay were measured as described; 10 units correspond to a 1 mm diameter clear zone around the sample well. Figures 1-6 show the results against five test organisms in units described as above. A synthetic protegrin (PG-1) containing two cystines (SPG-1) or PG-1 in linear form were used as controls.

Figure 1 shows the results for trans-parevin and tachytegrin with respect to *E. coli* both with and without the addition of 100 mM NaCl. Both of these peptides were slightly more effective than SPG-1 although slightly less effective than linear PG-1 in the absence of salt. However, in the presence of 100 mM NaCl, all four peptides were comparably effective.

Figure 2 shows the results of the same determination with respect to *L. monocytogenes*. With respect to this organism, all four peptides were roughly similarly effective in the absence of salt; the presence of 100 mM NaCl, however, greatly reduced the effectiveness of linear PG-1. The remaining three peptides remained effective under these conditions.

Figure 3 shows the results of the same experiment using *C. albicans* as the target organism. All four peptides were comparably effective in the absence of salt; again, the effectiveness of linear PG-1 was greatly reduced in the presence of 100 mM NaCl, while the remaining three peptides maintained their effectiveness under these conditions.

Figures 4-6 show the results of similar experiments using, as test peptides, the two isomers of parevin, trans-parevin (hairpin) and cis-parevin (cloverleaf). SPG-1 was used as a control. As shown in Figure 4, the two parevins were comparably effective in the absence of salt and both were more effective than SPG-1. In the presence of 100 mM NaCl, all three peptides maintained their effectiveness and were comparable.

Figure 5 shows results of the same experiment conducted with *B. subtilis* as target organism. Again, both forms of parevin were comparably effective and both were slightly more effective than SPG-1; in the presence of 100 mM NaCl, all three peptides remained effective antimicrobials and had about the same activity.

The results obtained with respect to *S. typhimurium* are also similar, as shown in Figure 6. Again, the two parevins were more effective than SPG-1 in the absence of salt and all three peptides had comparable effectiveness when 100 mM NaCl was added.

### Example 3

#### Ability to Bind Endotoxin

The compounds of the invention are tested for their ability to bind the lipid polysaccharide (LPS) of the gram-

negative bacterium *E. coli* strain 0.55B5, using the *Limulus* amebocyte lysate (LAL) test for endotoxins conducted in the presence and absence of the test compounds. The test is conducted using the procedure described in Sigma Technical  
5 Bulletin No. 210 as revised in December 1992 and published by Sigma Chemical Company, St. Louis, MO.

The LAL test is based on the ability of LPS to effect gelation in the commercial reagent E-Toxate® which is prepared from the lysate of circulating amebocytes of the  
10 Horseshoe Crab *Limulus polyphemus*. As described in the technical bulletin, when exposed to minute quantities of LPS, the lysate increases in opacity as well as viscosity and may gel depending on the concentration of endotoxin. The technical bulletin goes on to speculate that the mechanism  
15 appears analogous to the clotting of mammalian blood and involves the steps of activation of a trypsin-like preclotting enzymes by the LPS in the presence of calcium ion, followed by enzymic modifications of a "coagulogen" by proteolysis to produce a clottable protein. These steps are  
20 believed tied to the biologically active or "pyrogenic" portion of the molecule. It has been shown previously that detoxified LPS (or endotoxin) gives a negative LAL test.

The test compounds are used at various concentrations from 0.25 ug-10 ug in a final volume of 0.2 ml and the test  
25 mixtures contained LPS at a final concentration of 0.05 endotoxin unit/ml and E-Toxate™ at the same concentration. The test compounds are incubated together with the LPS for 15 minutes before the E-Toxate™ is added to a final volume after E-Toxate™ addition of 0.2 ml. The tubes are then incubated  
30

for 30 minutes at 37°C and examined for the formation of a gel.

In a follow-up experiment, the concentration of LPS is varied from 0.05-0.25 endotoxin units (E.U.).

5

#### Example 4

##### Antimicrobial Activity Under Conditions

##### Suitable for Treatment of the Eye

Contact lens solutions are typically formulated with  
10 borate buffered physiological saline and may or may not  
contain EDTA in addition. The compounds of the invention are  
tested generally in the assay described in Example 2 wherein  
all underlay gels contain 25 mM borate buffer, pH 7.4, 1%  
(v/v) trypticase soy broth (0.3 ug/ml TSB powder) and 1%  
15 agarose. Additions include either 100 mM NaCl, 1 mM EDTA or  
a combination thereof. Other test compounds used as controls  
are the defensin NP-1 and lysozyme, and dose response curves  
are determined.

20

#### Example 5

##### Preparation of *Enantio* Trans-parevin

Using standard solid phase techniques, a peptide having  
the amino acid sequence of trans-parevin, but wherein every  
amino acid is in the D form is prepared. This form is tested  
25 against *E. coli*, *L. monocytogenes*, *C. albicans* and other  
microbes in the absence and presence of protease and  
otherwise as described for the radiodiffusion assay in  
agarose gels set forth in Example 2.

30

## Example 6

### Activity Against STD Pathogens

The compounds of the invention are tested for antimicrobial activity against various STD pathogens. These include HIV-1, *Chlamydia trachomatis*, *Treponema pallidum*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, Herpes simplex type 2, Herpes simplex type 1, *Hemophilus ducreyi*, and Human papilloma virus. The results are provided in a form wherein "active" means that the peptide is effective at less than 10 ug/ml; moderately active indicates that it is active at 10-25 ug/ml; and slightly active means activity at 25-50 ug/ml. If no effect is obtained at 50-200 ug/ml the compound is considered inactive.

The compounds of the invention are tested for their antimicrobial activity against *Chlamydia* using the "gold standard" chlamydial culture system for clinical specimens described by Clarke, L.M. in Clinical Microbiology Procedures Handbook II (1992), Isenberg, H.T. Ed. Am. Soc. Microbiol. Washington, D.C.; pp. 8.0.1 to 8.24.3.9.

In the assays, *C. trachomatis* serovar L2 (L2/434Bu) described by Kuo, C.C. et al. in Nongynococcal Urethritis and Related Infections (1977), Taylor-Robinson, D. et al. Ed. Am. Soc. Microbiol. Washington, D.C., pp. 322-326 is used. The seed is prepared from a sonicated culture in L929 mouse fibroblast cells, and partially purified by centrifugation. Since host protein is still present in the seed aliquots, each seed batch is titered at the time of preparation with serial ten-fold dilutions to  $2 \times 10^{-9}$ . The seed containing  $9.2 \times 10^6$  IFU/ml is thawed quickly at 37°C and diluted to  $10^{-2}$  with sucrose/phosphate salts/glycine to produce IFU of about

200 after room temperature preincubation and to dilute background eukaryotic protein.

In the initial assays, the peptides to be tested are prepared as stock solutions in 0.01% glacial acetic acid.

- 5 100 ul of the diluted chlamydial seed are aliquoted into 1.5 ml eppendorf tubes and 200 ul of the antibiotic peptide was added per tube. Aliquots of the peptide stock (and controls) are incubated with the seed at room temperature for one hour, two hours and four hours. About 10 minutes before the end of
- 10 each incubation period, maintenance media are aspirated from the McCoy vials in preparation for standard inoculation and culture. Culture is then performed in the presence and absence of the peptides; in some cases, the peptides are added to final concentration in the culture media in addition
- 15 to the preculture incubation. The test is evaluated microscopically.

In another series of experiments, various concentrations of tachytegrin (1 ug, 12.5 ug, 25 ug and 50 ug) are used in the two-hour preincubation.

- 20 The effect of the presence of serum is also tested. The *Chlamydia* seed is preincubated for two hours with and without 10% FBS and also with or without test compound at 25 ug.

- The experiments are repeated but adding 25 ug of compound after the start of the chlamydial culture, i.e.,
- 25 after centrifugation and final medium mix and one hour into the beginning of the 48-hour culture period. Finally, the compound (at 25 ug) is added to the chlamydial seed and the mix then immediately cultured.



The effect of serum is particularly important since for a topical agent to be effective in combatting *Chlamydia* infection, it must act in the presence of serum.

In addition, there are several mouse-based models for *Chlamydia* infection which can be used to assess the efficacy of the tachytegrins. These include those described by Patton, D.L. et al. in Chlamydial Infections (1990) Bowie, W.R. et al. Eds. Cambridge University providing a source of complement. Ten ul of a suspension of *T. pallidum* containing about  $5 \times 10^7$ /ul organisms is added to each tube and the mixtures with the appropriate peptides are incubated at 34°C under 95% N<sub>2</sub> and 5% CO<sub>2</sub>. At time zero, just prior to incubation, 4 hours and 16 hours, 25 randomly selected organisms are examined for the presence or absence of motility. The 50% immobilizing end point (IE<sub>50</sub>) is calculated to indicate the concentration needed to immobilize 50% of the spirochetes. Tachyplesin IE<sub>50</sub>s are 5.231 ug and 2.539 ug for 0 and 4 hours, in contrast to HNP and NP preparations which show little immobilizing ability.

For Herpes Simplex Virus, using viral stocks prepared in VERO cells, grown in minimal essential medium (MEM) with 2% fetal calf serum, the effect of various peptides on HSV 1 MacIntyre strain, a pool of ten clinical HSV 1 isolates, HSV-2G, and a pool of ten clinical HSV 2 isolates, all sensitive to 3 uM acyclovir are tested. Two fibroblast cell lines, human W138 and equine CCL57, are used as targets and tests are done by direct viral neutralization and delayed peptide addition.

In the direct neutralization format, the virus is preincubated with the peptides for 90 min before it is added

to the tissue culture monolayers. In the delayed peptide addition format, the virus is added and allowed 50 min to adsorb to the target cells, then the monolayers are washed and peptides are added for 90 min. Finally, the monolayer is  
5 washed to remove the peptide and the cells are fed with peptide-free MEM and cultured until the untreated infected monolayers exhibit 4+ cytopathic effect (CPE) (about 60 hours).

For *Trichomonas vaginalis*, strain C1 (ATCC 30001) is  
10 grown as described by Gorrell, T.E. et al., Carlsberg Res Comm (1984) 49:259-268. In experiments performed in RPMI + 1% heat-activated fetal calf serum, within a few minutes after exposure to 50 ug/ml PG-1, *T. vaginalis* (heretofore vigorously motile) becomes stationary. Soon thereafter, the  
15 organisms become permeable to trypan blue, and, over the ensuing 15-30 minutes, lyse. As expected, such organisms fail to grow when introduced into their customary growth medium (Diamond's medium). Organisms exposed to 25 ug/ml of PG-3 retain their motility.

20

#### Example 7

##### Antiretroviral Activity

The invention compounds are tested for antiviral activity against strains of HIV using the method described in  
25 Miles, S.A. et al., Blood (1991) 78:3200-3208. Briefly, the mononuclear cell fraction is recovered from normal donor leukopacs from the American Red Cross using a Ficoll-hypaque density gradient. The mononuclear cells are resuspended at  $1 \times 10^6$  cells per ml in RPMI 1640 medium with 20% fetal bovine  
30 serum, 1% penn/strep with fungizone and 0.5% PHA and

incubated 24 hours at 37°C in 5% CO<sub>2</sub>. The cells are centrifuged, washed and then expanded for 24 hours in growth medium.

5 Non-laboratory adapted, cloned HIV<sub>JR-CSF</sub> and HIV<sub>JR-FL</sub> are electroporated into the human peripheral blood mononuclear cells prepared as described above. Titers are determined and in general, multiplicities of infection (MOI) of about 4,000 infectious units per cell are used (which corresponds to 25-40 picograms per ml HIV p24 antigen in the supernatant).

10 In the assay, the HIV stocks prepared as above are diluted to the correct MOI and the PBM are added to 24 well plates at a concentration of  $2 \times 10^6$  per ml. One ul total volume is added to each well. The peptide to be tested is added in growth medium to achieve the final desired  
15 concentration. Then the appropriate number of MOI are added. To assay viral growth, 200 ul of supernatant is removed on days 3 and 7 and the concentration of p24 antigen is determined using a commercial assay (Coulter Immunology, Hialeah, Florida). Controls include duplicate wells  
20 containing cells alone, cells plus peptide at 5 ug/ml cells with virus but not peptide and cells with virus in the presence of AZT at  $10^{-5}$  M -  $10^{-8}$  M.

The time of addition of peptide can be varied. Cells pretreated for 2 hours prior to addition of virus, at the  
25 time of addition of virus, or 2 hours after infection show antiviral activity for the peptide.

## Example 8

### Preparation and Activity of Tachytegrins

Several illustrative tachytegrins were synthesized as described in Example 1 and tested for activity against

5 *Staphylococcus aureus* (MRSA), *Pseudomonas* (Psa), VREF, *Candida* and *E.coli* as described in Example 2. The results shown in Table 1 as minimal inhibitory concentration (MIC) in ug/ml were obtained with the C-terminal amidated forms except for the last two which were tested as the free acids, as

10 indicated by \*. In all peptides in Table 1, X is MeGly.

15

20

25

30

TABLE 1

SEQUENCE	SEQ ID NO:77	MRSA	Psa	VREF	Candida	E. Coli
RGGCLRYAVPRFAVRVCR	SEQ ID NO:77	>128	0.05			
RGGCLRYTKPKFTVRVCR	SEQ ID NO:78					
RGGCLRYAVGRFAVRVCR	SEQ ID NO:79					
RGGCLRYARXRFAVRVCR	SEQ ID NO:80	>32	5.7			
RGFCLRYTVPRFTVRFCVR	SEQ ID NO:81	1.88	0.57			0.99
RGFCLRYKVGRFKVRFCVR	SEQ ID NO:82	>64	2.7			
RGFCLRYXVGRFXVRFCVR	SEQ ID NO:83					
RGGCLRYCRRPRFCVRVCR	SEQ ID NO:53	9.8	0.18		9.68	0.2
RGGCLRYCRRRRCVVGCR	SEQ ID NO:54	53.3	3.3	4		4
RGVCLRYCRRFCVRLCR	SEQ ID NO:55	8	2			
RGRVCLRYCRRFCVRLCFR	SEQ ID NO:56	6.7	1			
RWRVCLRYCRRFCVRLCLR	SEQ ID NO:57	4	4			
RGWRVCLKYCRRFCVVKLCLR	SEQ ID NO:58					
RGGRVCLRYCRRGKFCVRLCLR	SEQ ID NO:59	8	0.75			
RGGCLRYAVGRFAVRVCR*	SEQ ID NO:77	>32		5.3		
RGFCLRYXVGRFXVRFCVR*	SEQ ID NO:83	>32		12		

5

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